The Transmembrane Domain of a Carboxyl-terminal Anchored Protein Determines Localization to the Endoplasmic Reticulum*

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UBC6 is a C-terminal membrane-anchored (type IV) protein, native to *Saccharomyces cerevisiae*, where it is found in the endoplasmic reticulum. When expressed in mammalian cells, this novel ubiquitin-conjugating enzyme also localizes to the endoplasmic reticulum. UBC6 lacks a lumenal domain and contains no known endoplasmic reticulum retention signals. Analysis of chimeric proteins in which the cytosolic domain of UBC is linked to a heterologous transmembrane domain, or in which the UBC6 transmembrane domain is appended to an unrelated soluble protein, led to the determination that the transmembrane domain of UBC6 plays a dominant role in its compartmental localization. The basis for the transmembrane domain-mediated subcellular targeting of UBC6 was evaluated by lengthening the wild type UBC6 hydrophobic segment from 17 to 21 amino acids, which resulted in re-targeting to the Golgi complex. A further increase in length to 26 amino acids allowed this modified protein to traverse the secretory pathway and gain expression at the plasma membrane. These findings are consistent with models in which, in the absence of dominant cytosolic or lumenal targeting determinants, proteins may be sorted within the secretory pathway based on interactions between their transmembrane domains and the surrounding lipid bilayer.

Fundamental to understanding the secretory pathway is an elucidation of the molecular basis by which organelles of this system are formed, and a determination of the means by which steady state levels of resident proteins and lipid are maintained. As the default pathway for the vectorial flow of proteins leads from the ER[1] to the plasma membrane (1, 2), mechanisms are necessary to retain resident proteins within intracellular organelles. Several specific signals have been identified for the retention/retrieval of ER proteins. Retrieval from the Golgi of soluble ER resident proteins is mediated by recognition of the tetra-amino acid sequence "KDEL" by a specific receptor (3). For transmembrane proteins, intracytoplasmic sequences play a role in ER retention/retrieval. A di-lysine motif at the C terminus of type I proteins allows for retrieval from the Golgi to

the ER in a coatomer-dependent manner (4–7); a di-arginine N-terminal motif may play an analogous role for type II proteins (8), and a tyrosine-based motif also functions as a cytoplasmic ER retention signal (9). Proteins can also be indirectly retained by interactions with ER resident proteins. For example, lumenal chaperones including BiP, calnexin, and calreticulin interact with newly synthesized proteins in the ER lumen and mediate transient or stable retention of proteins that are devoid of intrinsic ER retention/retrieval sequences (10).

UBC6 is a 250-amino acid ER resident protein that has no known ER retention/retrieval signal (11). This membrane-anchored *Saccharomyces cerevisiae* ubiquitin-conjugating enzyme is implicated in the degradation of a transcriptional repressor (MATα2) and of a temperature-sensitive mutant of the ER translocator (Sec 61). This has led to speculation that UBC6, and perhaps mammalian homologues, plays a role in ubiquitin-mediated degradation of ER proteins (11, 12). UBC6 is a member of the C-terminal anchored, or type IV, class of transmembrane proteins. These proteins are characterized by hydrophobic segments close to, or at, their C termini, precluding the co-translational SRP-dependent membrane insertion characteristic of most transmembrane proteins (13, 14). In this study we evaluate the basis for ER retention of UBC6 and establish that the C-terminal hydrophobic membrane anchor plays a dominant role in determining the localization of UBC6 within the secretory pathway.

MATERIALS AND METHODS

DNA Recombinant Procedures and Transfections—A cDNA encoding UBC6 in pTX8 was a gift from S. Jentsch (University of Heidelberg). A 1.02-kilobase fragment was subcloned into pGEM7z⁺ (Promega, Madison WI) and subsequently transferred to pSVL (Pharmacia Biotech Inc.). UBC6 with an N-terminal myc epitope tag and four-amino acid linker (see Fig. 1) was generated by PCR using appropriate oligonucleotides[2] and UBC6 in pGEM7z⁺ as template (Fig. 1). PCR was carried out with conditions of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min for 30 cycles. The product was cloned into pGEM7z⁺, and subcloned into pSVL from *Xho*I to *Bam*HI (pSVL-*m*UBC6). In a similar fashion, UBC6 with a C-terminal myc tag was generated by PCR and subcloned into pSVL. To generate variants in the C terminus of *m*UBC6, an oligonucleotide corresponding to bases 517–540 of UBC6 (U517-40) was paired with antisense (3′) oligonucleotides encoding the desired C-terminal modifications in the PCR (Fig. 1). Products were cut with *Xba*I and *Bam*HI and cloned into pSVL-*m*UBC6 that had been cut with *Xba*I and *Bam*HI. A construct encoding *m*UBC6ᵀᶠᴿ was generated by a two-step PCR method (15). The final product corresponds to the cytoplasmic coding region of *m*UBC6 linked to amino acids 61–90 of the TfR (Fig. 1). Green fluorescence protein (16) with a recognition site for 9E10 at the N terminus and the C-terminal 18 amino acids of UBC6 at its C terminus (*m*GFPᵁᴮᶜ⁶) was also generated by two-step PCR.

Myc-tagged ZAP-70 was from L. Samelson and R. Wange (National Institutes of Health) (17). Tac-E19 has been previously described (18); plasmids encoding mannosidase II and the interleukin-2 receptor α

[1] The abbreviations used are: ER, endoplasmic reticulum; SRP, signal recognition protein; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TfR, transferrin receptor; TMD, transmembrane domain; PCR, polymerase chain reaction.

[2] Sequences of oligonucleotides are available on request.

subunit (Tac) were from N. Cole (National Institutes of Health) (19), and W. Leonard (National Institutes of Health) (20), respectively.

Cells and Antibodies—COS-7 cells (American Tissue Culture Collection, number CRL1651, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD) supplemented with 8% fetal bovine serum (complete medium). Hybridomas secreting monoclonal antibodies 9E10 (anti-myc) (21) and 7G7 (anti-interleukin-2 α subunit) (22) were obtained from the American Tissue Culture Collection and from D. Nelson (National Institutes of Health), respectively. Polyclonal rabbit antisera raised against UBC6 (11), anti-interleukin-2 receptor α subunit (23), mannosidase II (24), and ribophorin I (25) were from T. Sommer and S. Jentsch (University of Heidelberg), W. Leonard (National Institutes of Health), K. Moremen (Whitehead Institute), and G. Kreibich (New York University).

Immunofluorescence Microscopy—Immunofluorescence on transfected COS-7 cells was carried out as described (26) using secondary antibodies obtained from Jackson ImmunoResearch (West Grove, PA) conjugated to indocarbocyanine (Cy3), fluorescein, or rhodamine. For co-localization experiments, *m*UBC6 constructs were co-transfected with known markers. After incubation with primary mouse and rabbit antibodies, double staining was carried out with fluorescein-labeled anti-mouse and rhodamine-labeled anti-rabbit secondary antibodies. Transfection efficiencies were generally 20–30%, in all experiments over 200 transfected cells were examined for each field shown. For time course studies (Fig. 7), cells were transfected by electroporation using a Bio-Rad Gene Pulsar, with conditions of 240 V and 500 microfarads with 5×10^6 cells in 0.2 ml of complete media. Electroporated cells were plated on coverslips and processed at the indicated times. Brefeldin A (Epicentre Technologies, Madison, WI) was used at a final concentration of 1 μg/ml.

Electrophoretic Protein Analyses—For metabolic labeling studies, cells were transfected with 20 μg of DNA by calcium phosphate precipitation (27). After 24 h, cells were removed from plates using Versene (Biofluids) and labeled in 1 ml of methionine-free complete medium containing 0.3 mCi/ml of [^{35}S]methionine (Tran^{35}S-label, ICN Radiochemicals, Irvine, CA) for 30 min at 37 °C. Cells were then washed and resuspended in complete medium. At designated time points cells were collected and washed three times in ice-cold PBS. Detergent lysis of cell pellets, immunoprecipitation, and SDS-PAGE have all been described (28). Gels were fixed followed by impregnation with Enlightning (DuPont NEN) and autoradiography at −70 °C.

For analysis of membrane and cytosolic fractions, transfected COS-7 cells were resuspended in 2 ml of 30% normal tonicity PBS supplemented with protease inhibitors (28). After 10 min at 4 °C, cells were disrupted with 40 strokes of a Dounce homogenizer, and tonicity was immediately restored with hypertonic PBS. After removal of nuclei and unbroken cells at $300 \times g$, membranes were pelleted at $100,000 \times g$. Membranes were solubilized in Triton X-100 containing buffer (28), and membrane and cytosolic samples were immunoprecipitated, resolved on SDS-PAGE, transferred to nitrocellulose, and subjected to Western blotting with biotin-labeled 9E10 followed by detection using streptavidin-horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL) and enhanced chemiluminescence (Amersham Life Sciences).

Subcellular Fractionation—Following electroporation with 25 μg of plasmid (as above), 10^7 COS-7 cells were plated into duplicate 100-mm dishes. After 48 h, plates were rinsed twice with Buffer A (0.25 M sucrose, 10 mM triethanolamine, 1 mM EDTA, pH 7.4) and harvested by gentle scraping into 5 ml of homogenization buffer (85% Buffer A (v/v) and 15% of Buffer B (10 mM Tris, pH 7.4, 5 mM KCl, 1 mM EDTA, 128 mM NaCl)). Cells were spun at $300 \times g$ and resuspended in 0.3 ml of homogenization buffer plus protease inhibitors (28), followed by homogenization at 4 °C by 12 passages through a 25-gauge needle attached to a 1-ml syringe. Post-nuclear supernatants were prepared by two consecutive centrifugations at $1000 \times g$. Supernatants were loaded on pre-formed 0–26% Optiprep (Iodixanol), linear gradients that had been pre-cooled to 4 °C. Gradients were spun for 115 min at 41,000 rpm at 4 °C in a SW41 rotor in a Beckman ultracentrifuge. Twenty fractions (600 μl each) were recovered by bottom piercing using a Beckman Fraction Recovery System. For immunoblotting, fractions were diluted to 1.2 ml with Buffer B, and membranes were pelleted in a Beckman High Speed Microfuge at 4 °C in a TLA45 rotor at $125,000 \times g$ for 60 min. Pellets dissolved in SDS-PAGE sample buffer were subject to electrophoresis and immunoblotting.

Gradients were formed by underlayering 6 ml of 26% Optiprep solution (26% Iodixanol (Nycomed, Oslo, Norway) in Buffer B) under 6.5 ml of Buffer B containing added protease inhibitors in SW41 tubes (Beckman) and inserting into a Gradient Master (Biocomp Instruments,
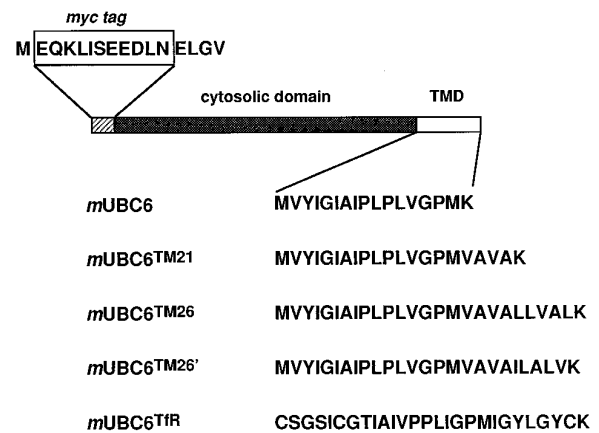

FIG. 1. **Modifications of UBC6.** Myc-tagged UBC6 (*m*UBC6) and derivatives thereof with altered TMDs are represented schematically. The sequence recognized by the monoclonal antibody 9E10 is *boxed*, and the tetra amino acid sequence "ELGV" serves as a linker to the body of UBC6.

Fredericton, Canada) and rotating at 15 rpm for 2.22 min at 1.5° above the horizontal angle. The resultant gradient was from bottom to top 26 to 0% Iodixanol. Cell surface biotinylation (29) and the galactosyltransferase assay (30) were carried out as described.

RESULTS

The Transmembrane Domain of UBC6 Determines Its Subcellular Location—To determine the subcellular location of UBC6 when expressed in mammalian cells, a construct was generated encoding UBC6 with a myc-epitope tag at its N terminus (*m*UBC6) (Fig. 1). This tag allows for recognition by an anti-myc-peptide monoclonal antibody, 9E10 (21). When *m*UBC6 was transiently expressed in COS-7 cells and examined by immunofluorescence microscopy using 9E10 as the primary antibody, a lacy pattern characteristic of the ER was seen (Fig. 2A). Untagged UBC6 (assessed using anti-UBC6 rabbit sera) and C-terminal myc-tagged UBC6 exhibited similar ER patterns (not shown). Since interpretation of results with the N-terminal epitope tag are not complicated by issues related to the introduction of a lumenal domain, *m*UBC6 was used as the basis for the construction of additional epitope-tagged constructs (Fig. 1).

As UBC6 has no intralumenal domain, cytosolic and/or transmembrane domains (TMDs) must play an essential role in its localization. To evaluate this, the TMD of the human transferrin receptor (TfR) (31) was substituted for the native TMD of UBC6 (*m*UBC6^{TfR}) (Fig. 1). Structurally, the TfR is a typical type II protein, normally targeted to the plasma membrane where it undergoes endocytosis and re-cycling to the cell surface (32). When cells expressing *m*UBC6^{TfR} were assessed by immunofluorescence microscopy, the pattern found was dramatically different from that of *m*UBC6, as *m*UBC6^{TfR} was prominently expressed at the plasma membrane, although staining of intracellular membranes was still apparent (Fig. 2B). This indicates that a heterologous TMD is sufficient to re-target UBC6 to the plasma membrane and suggests that the TMD plays a dominant role in determining the subcellular location of UBC6. To ascertain whether the UBC6 TMD is sufficient to direct a heterologous protein to the ER, a construct was generated encoding the UBC6 TMD appended to the C terminus of myc-tagged green fluorescence protein (16) (*m*GF-P^{UBC6}). Staining with 9E10 revealed a pattern consistent with ER localization (Fig. 2C). Thus, it would appear that the TMD of UBC6 is necessary for targeting of UBC6 to the ER and also capable of targeting a heterologous protein to ER membranes.

Retargeting to the Golgi and Plasma Membrane by Increasing the TMD Length—The TMD of UBC6 is only 17 amino acids
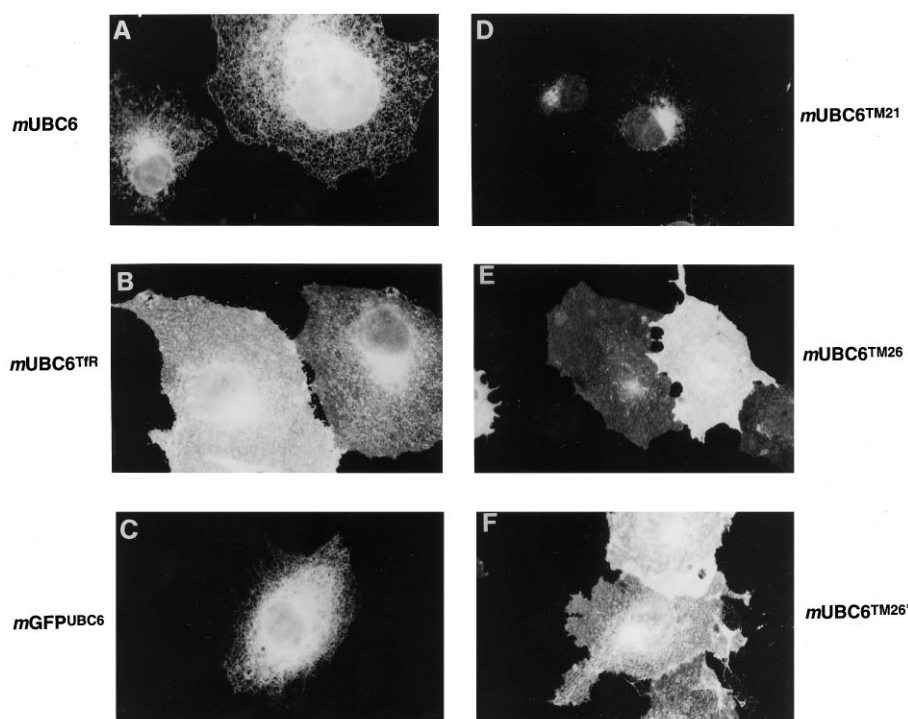
Fig. 2. **The TMD of UBC6 determines its subcellular location.** Constructs encoding mUBC6 (*A*), mUBC6^{TfR} (*B*), mGFP^{UBC6} (*C*), mUBC6^{TM21} (*D*), mUBC6^{TM26} (*E*), or mUBC6$^{TM26'}$ (*F*) were transiently expressed in COS-7 cells by calcium phosphate precipitation. Cells were analyzed by immunofluorescence microscopy 40 h after transfection using the monoclonal anti-myc peptide 9E10, followed by Cy3-conjugated donkey anti-mouse IgG. Shown are representative fields from multiple independent transfections. Transfection efficiencies were generally 20–30%.

in length, whereas the TfR has a predicted membrane-spanning segment of 28 amino acids. To determine if TMD length plays a role in the ER location of UBC6, the TMD of mUBC6 was lengthened by the insertion of four additional hydrophobic amino acids (VAVA) prior to the C-terminal lysine, for a total of 21 transmembrane residues (Fig. 1). When this modified molecule, mUBC6^{TM21} (Fig. 2*D*), was compared with mUBC6, a marked change in distribution was observed, with mUBC6^{TM21} found mainly in a discrete perinuclear region characteristic of the Golgi complex.

It has been proposed that the generally shorter TMDs of resident Golgi proteins relative to their plasma membrane counterparts is, at least in part, responsible for their subcellular localization (33). To address whether mUBC6^{TM21} could be retargeted from the Golgi to the plasma membrane by further lengthening of the TMD, its hydrophobic segment was increased by five more amino acids (LLVAL), for a total of 26 transmembrane residues (Fig. 1). When expressed transiently, this protein, mUBC6^{TM26}, exhibited a pattern similar to that seen with mUBC6^{TfR}, consistent with expression both at the plasma membrane and in intracellular compartments (Fig. 2*E*). To ensure that this plasma membrane expression was not due to the incidental generation of a plasma membrane targeting sequence, a second construct encoding a different five amino acid addition (ILALV) was assessed (mUBC6$^{TM26'}$). As is evident, the pattern found with mUBC6$^{TM26'}$ (Fig. 2*F*) is similar to that found with mUBC6^{TM26}.

The distributions of the various mUBC6 constructs were confirmed by co-localization studies with known ER, Golgi, and plasma membrane markers (Fig. 3). mUBC6, bearing the wild type TMD, exhibits a pattern almost indistinguishable from the ER-retained protein, Tac-E19 (Fig. 3, *A* and *B*); mUBC6^{TM21} co-localizes with a Golgi marker, mannosidase II (Fig. 3*C* and *D*); and finally, the staining found with mUBC6^{TM26} is strikingly similar to that seen with the Tac antigen (the interleukin-2 receptor α subunit) (Fig. 3, *E* and *F*), a protein known to be expressed on the cell surface (18, 20, 24). The TMD-mediated re-targeting of mUBC6 was further evaluated by subcellular fractionation using Iodixanol-based linear density gradients. In these studies, mUBC6 was found predom-

inantly in the denser fractions of the gradients, where it co-migrated with an endogenous ER protein, ribophorin I (Fig. 4, compare *panels A* and *B*). mUBC6^{TM26}, on the other hand, exhibited a more heterogeneous distribution, with over 60% of the recovered protein in less dense (post-ER) fractions, consistent with trafficking of this protein from the ER through the Golgi to the plasma membrane.

To confirm that all of the UBC6-based proteins are membrane-bound, cytosolic and membrane fractions were prepared from cells expressing epitope-tagged UBC6 constructs and immunoprecipitated with 9E10 (Fig. 5). The mUBC6-derived proteins were found almost exclusively in the membrane fractions. In contrast, a myc-tagged non-transmembrane tyrosine kinase, ZAP-70 (34), was found largely in the cytosolic fraction. To determine if the membrane orientation of UBC6 is maintained when the transmembrane domain is lengthened to 26 amino acids, cells that were fixed and permeabilized prior to staining with 9E10 were compared with those that were not permeabilized. Permeabilization and fixation was required for recognition of mUBC6^{TM26} by 9E10, while Tac was recognized by an antibody directed against its ectodomain regardless of permeabilization (not shown). Thus, lengthening the transmembrane domain of UBC6, while altering its subcellular distribution, does not change its membrane association or its orientation.

The most straightforward explanation for these findings is that the length of the hydrophobic transmembrane segment is responsible for determining the subcellular location of UBC6. An alternative possibility is that proteins bearing the wild type TMD are rapidly degraded after synthesis and therefore only seen in the ER, whereas those with heterologous or modified TMDs are more stable and consequently visualized at later stages during their progression through the secretory pathway. To address this, the relative half-lives of the mUBC6 constructs were examined by pulse-chase metabolic labeling using [^{35}S]methionine. As shown (Fig. 6), mUBC6, mUBC6^{TM21}, mUBC6^{TM26}, and mUBC^{TfR} were all stable for at least 6 h. In contrast, mUBC6^{TM14}, which has only a 14-amino acid hydrophobic stretch, was lost with a $t_{1/2}$ of less than 2 h. These results establish that differences in compartmental distribution among variants of mUBC with different length TMDs cannot

FIG. 3. **Co-localization with known cellular markers.** COS-7 cells were co-transfected with constructs encoding either *m*UBC6 and a known ER marker, *Tac-E19* (*A* and *B*); *m*UBC6^{TM21} and a known Golgi marker, mannosidase II (*C* and *D*); or with *m*UBC6^{TM26} and *Tac* (a cell surface protein) (*E* and *F*). Fixed and permeabilized cells were incubated with 9E10, which recognizes the epitope-tagged *m*UBC6, *m*UBC6^{TM21}, and *m*UBC6^{TM26} and with rabbit polyclonal antisera directed against each of the appropriate markers. Cells were then incubated with fluorescein-conjugated donkey anti-mouse IgG and rhodamine-conjugated donkey anti-rabbit IgG. The subcellular locations of the *m*UBC6-derived proteins are demonstrated in *A, C,* and *E* using 488 nM excitation (fluorescein), whereas *B, D,* and *F* are the corresponding fields evaluated for the known markers using 568 nM excitation (rhodamine). The fields shown are representative of over 300 transfected cells analyzed in two independent experiments.
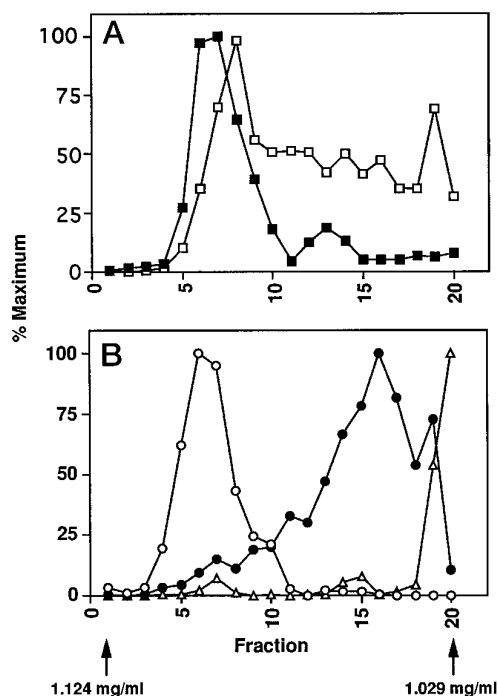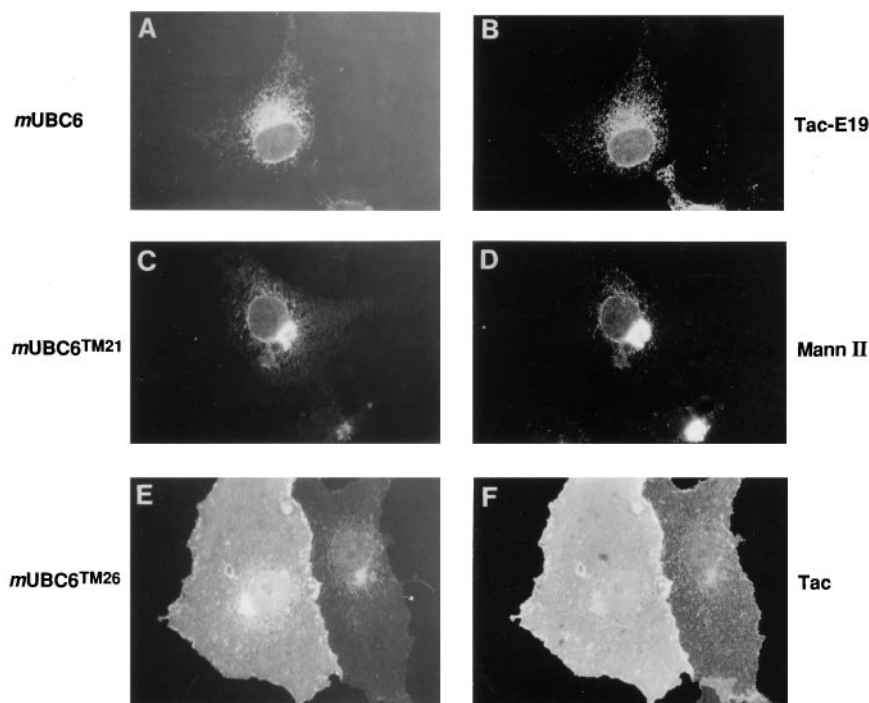

FIG. 4. **Subcellular fractionation of mUBC6 and mUBC^{TM26}.** COS-7 transfected with either *m*UBC6 (■) or *m*UBC6^{TM26} (□) were separated by differential centrifugation as described under "Materials and Methods," and fractions were analyzed by immunoblotting with 9E10 (*A*). Shown for comparison are the distribution of ER (ribophorin I, ○), Golgi (galactosyltransferase, ●), and plasma membrane (biotinylated cell surface proteins, △) markers (*B*). The densities at the *top* and *bottom* of the gradient are indicated. For each distribution, results are presented normalized to the maximum value.

FIG. 5. **Localization of mUBC6 proteins to membrane fractions.** Membrane (*M*) (*lanes 1, 3, 5, 7,* and *9*) and cytosolic (*C*) (*lanes 2, 4, 6, 8,* and *10*) fractions of COS-7 cells transfected with either *m*UBC6 (*lanes 1* and *2*), *m*UBC6^{TM21} (*lanes 3* and *4*), *m*UBC6^{TM26} (*lanes 5* and *6*), *m*UBC6^{TfR} (*lanes 7* and *8*), or *m*ZAP-70 (*lanes 9* and *10*) (17) were immunoprecipitated with 9E10 and resolved on 12% SDS-PAGE followed by immunoblotting with biotinylated 9E10 and developed with streptavidin-conjugated horseradish peroxidase and enhanced chemiluminescence (see "Materials and Methods" for details). The small amount of ZAP-70 seen in the membrane fraction (*lane 9*) may be a consequence of SH2-mediated associations with membrane proteins.

be accounted for by differences in their relative stabilities.

Plasma Membrane-targeted UBC6 Traverses the Secretory Pathway—Most integral membrane proteins of the secretory pathway gain entry into ER membranes via a co-translational, SRP-dependent mechanism and are then either retained in the ER or transported to their final destinations (14, 35, 36). While substantially less is known regarding the post-translational
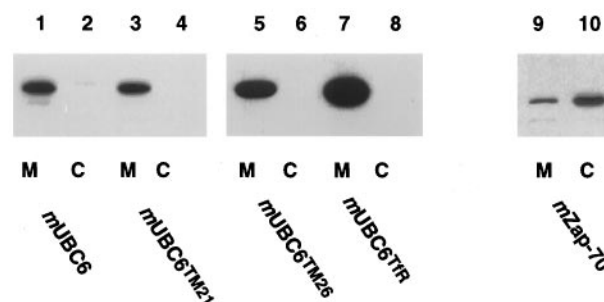
insertion of C-terminal anchored proteins (13), there are at least two examples where members of this family appear to be inserted into ER membranes (37, 38). The distribution of *m*UBC6^{TM26}, as assessed by immunofluorescence microscopy and subcellular fractionation (Figs. 3 and 4), is consistent with the idea that this protein reaches the plasma membrane after first being inserted into the ER. To evaluate this further, the time course of plasma membrane expression of *m*UBC6^{TM26} was assessed. Cells transfected by electroporation with *m*UBC6^{TM26} were examined 5 h after transfection (Fig. 7*A*) or incubated for an additional 5 h either without or with the addition of brefeldin A (Fig. 7, *B* and *C*). Brefeldin A is a fungal product that disrupts the Golgi complex, preventing newly synthesized proteins from reaching the plasma membrane (39–41). As shown, at 5 h after transfection, the pattern seen with *m*UBC6^{TM26} is consistent with distribution in the ER, with perhaps some Golgi staining as well. Incubation for an additional 5 h results in the development of plasma membrane staining that was not visible at 5 h and which was blocked by

Fig. 6. **Pulse-chase analysis of mUBC-derived proteins.** Cells transfected with *m*UBC6 (*lanes 1–3*), *m*UBC6[TM21] (*lanes 4–6*), *m*UBC6[TM26] (*lanes 7–9*), *m*UBC6[TfR] (*lanes 10–12*), or *m*UBC6[TM14] (*lanes 13–15*) were pulsed for 30 min with [35S]methionine and chased for either 0, 2, or 6 h in complete medium as indicated, followed by immunoprecipitation with 9E10 and resolution on 12% SDS-PAGE.
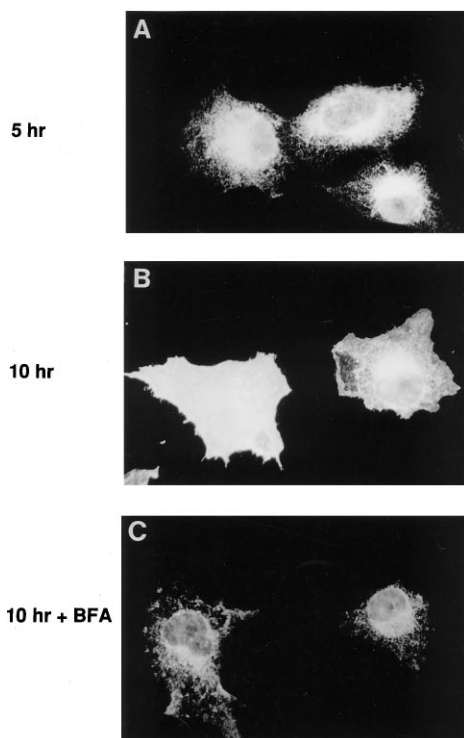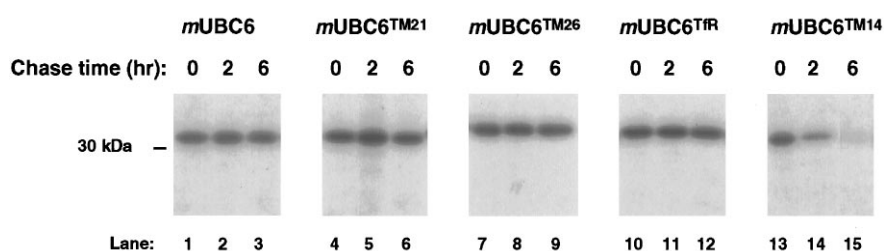

Fig. 7. **mUBC6[TM26] reaches the plasma membrane by traversing the secretory pathway.** Cells transfected with *m*UBC6[TM26] by electroporation were analyzed by immunofluorescence at 5 h after transfection (*A*) or 10 h after transfection (*B*). *C*, brefeldin A (*BFA*) (1 μg/ml) was added at 5 h after transfection, and cells were analyzed after a total of 10 h. Shown are representative fields from over 300 transfected cells that were analyzed.

brefeldin A. Similar findings were obtained with the type I plasma membrane protein Tac (not shown). These observations are consistent with the notion that C-terminal anchored proteins with long transmembrane domains, like proteins that gain access to the secretory pathway in an SRP-dependent fashion, are inserted into the ER and then sorted to their final destinations.

DISCUSSION

For most transmembrane proteins, hydrophobic signal peptides facilitate the SRP-dependent co-translational insertion of nascent proteins into the ER membrane (14, 35, 36). Type IV (C-terminal anchored) transmembrane proteins are an exception, as they are introduced into membranes in a post-translational, SRP-independent manner. Little is known regarding the mechanisms responsible for membrane insertion and intracellular localization of type IV proteins; however, their varied patterns of expression demonstrates that they encode sufficient information to confer targeting to specific intracellular addresses (13).

UBC6 is a type IV ER membrane protein that contains no canonical ER retention/retrieval sequences. Substitution of a heterologous 28-amino acid TMD re-targets UBC6 to the

plasma membrane, whereas ER localization is imparted to a soluble protein by addition of the wild type (17-amino acid) UBC6 TMD. Lengthening of the wild type UBC6 TMD to 21 amino acids allows for movement to the Golgi complex and a further increase to 26 amino acids facilitates traversal of the secretory pathway, leading to expression at the plasma membrane. Thus, it appears that for this type IV membrane protein, the length of the TMD plays a crucial role in targeting within the secretory pathway.

TMDs have been implicated in ER retention in other systems. Unassembled T cell antigen receptor α and β subunits are retained in the ER, at least in part, as a consequence of charged TMD amino acids (42). For a plasma membrane protein with a 19-amino acid TMD, introduction of charged residues into the hydrophobic segment leads to ER retention and, depending on the placement of the charge, to degradation (43). Similarly, for another cell surface-expressed chimera that has a charge within its TMD, shortening the predicted TMD from 23 to 17 amino acids leads to ER retention (44). Interestingly, the KDEL receptor contains an aspartic acid in one of its TMDs that is crucial for ER retrieval (45). The 20-amino acid TMD of microsomal cytochrome P450 targets to the ER, contains no charged residues, but does include several hydrophilic amino acids (46), and while this manuscript was being prepared, a study was published demonstrating that lengthening the 17-amino acid TMD of cytochrome b_5 by 5 amino acids resulted in a re-distribution from the ER to the cell surface (47).

TMDs play major roles in retention of proteins within the Golgi stack, as exemplified by galactosyltransferase, sialyltransferase, the avian coronavirus M protein, and other resident Golgi proteins (48–55). Whereas the range of TMD properties responsible for Golgi retention remains to be established, in general Golgi proteins have substantially shorter TMDs than their cell surface counterparts. This has led to the hypothesis that lipid composition-dependent differences in thickness and deformability between the lipid bilayers of the Golgi complex and those of the plasma membrane play a determining role in protein sorting (33). Since cholesterol is known to increase membrane thickness and to decrease deformability, and because its concentration in lipid bilayers increases as one proceeds outward through the secretory pathway (56), it has been implicated as being of consequence in "lipid-based" sorting between the Golgi and the plasma membrane (33, 55). In a lipid-based sorting model, proteins partition between the Golgi complex and the plasma membrane based on energetics; shorter hydrophobic segments preferentially distribute to the Golgi and longer ones to the thicker plasma membrane bilayer. The concept that TMD length determines distribution between the Golgi and the plasma membrane has recently been confirmed experimentally for both a Golgi and a plasma membrane protein (55).

The change in distribution associated with incremental increases in the length of the UBC6 TMD suggests that a lipid-based sorting mechanism may be operative not only between the Golgi and the plasma membrane but also between the cholesterol-poor, thinner/more deformable lipid bilayers of the

ER and those of the Golgi complex (56, 57). Based on correlations between charged TMD residues and ER retention, it seems likely that it is not only TMD length but also overall hydrophobicity that determines the ability of proteins to move out of the ER. ER retention of UBC6 and other proteins would represent the extreme situation where the TMDs are long enough for membrane insertion but too short and/or hydrophilic to allow these proteins to exit the ER and move to thicker/more rigid membranes. Lipid-based sorting does not preclude a role for TMD oligomerization in determining protein distribution. In fact, it has been suggested that oligomerization may improve the efficiency with which some proteins are retained in cisternae of the Golgi complex through TMD-mediated interactions (58). In other instances, such as with the charge-containing TMDs of the multi-subunit T cell antigen receptor, associations between TMDs of different subunits having opposing charges could affect the physical characteristics displayed to the hydrophobic milieu and thus facilitate the egress of assembled receptors from the ER, leading to their eventual expression at the cell surface (42).

The generation and maintenance of distinct protein populations among organelles of the secretory pathway is accomplished, at least in part, by specificity at the levels of vesicular transport and protein retention/retrieval. However, the proteins that form the bases for these targeting mechanisms must themselves be directed to specific compartments. Sorting mechanisms based on TMD length and hydrophobicity represent an efficient means for establishing such distributions as a consequence of physical interactions between TMDs and their surrounding lipid environment. In fact, the recent findings that several SNARE proteins are C-terminal anchored proteins with relatively short TMDs (37, 59–61) may be indicative of a primary role for TMDs in establishing these distributions. Our findings, placed in the context of the existing literature, make it reasonable to surmise that a gradient in the physical characteristics of the lipid bilayer exists within the secretory pathway, such that, in the absence of dominant lumenal or cytosolic associations, proteins distribute based on interactions between TMDs and the surrounding lipid environment. As C-terminal anchored proteins contain no lumenal domains, they represent potentially useful tools to further evaluate protein sorting among organelles of the secretory pathway.

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